ASSESSMENT OF PROTOCOL VARIABLES IN THE NTP EVALUATION OF CYTOTOTXICITY ASSAYS

Balb/C 3T3 Cells

I. What is the acceptable solvent concentration?

Two solvents, DMSO and EtOH, were assayed in the 3T3 assay to determine acceptable concentrations. Multiple exposure times were assessed since the final assay exposure time was not yet established. Various cell seeding concentrations were tested since these experiments were run concurrently with others which used to determine optimal seeding density.

Table 1.

EtOH

	Date	2%	1%	0.50%	Seeding Density
48h	2/26/02	58%	72%	100%	$9X10^3$ cells/ml
	2/26/02	49%	73%	102%	$4.5X10^3$ cells/ml
72h	2/26/02	67%	75%	105%	$9X10^3$ cells/ml
	2/26/02	68%	82%	108%	$4.5X10^3$ cells/ml

DMSO

	Date	2%	1%	0.5%	0.4%	0.3%	0.2%	0.1%	Seeding Density
24h	3/19/02		76%	91%	92%	99%	100%	101.6%	$2X10^4$ cells/ml
48h	2/26/02	25%	54%	83%					$9X10^3$ cells/ml
	2/26/02	27%	56%	78%					$4.5X10^3$ cells/ml
	3/19/02		116%	123%	122%	120%	117%	108.8%	$1X10^4$ cells/ml
72h	2/26/02	20%	52%	86%					$9X10^3$ cells/ml
	2/26/02	19%	56%	93%					$4.5X10^3$ cells/ml
	3/19/02		58%	89%	102%	102%	112%	110.1%	$5X10^3$ cells/ml

We concluded from these experiments that 0.5% EtOH was the optimal EtOH concentration (little to no toxicity), and that 0.5% was probably acceptable for DMSO as a trade-off between slight toxicity and ability to test chemicals to higher does levels.

From about the middle of March on, we used 0.5% in all of our experiments where DMSO was called for as a solvent. This gave us a number of

opportunities to further determine the toxicity of DMSO by comparing the solvent control wells with the media control wells in the same experiment.

Table 2.

DMSO			
Date & Exposure Time	OD Assay Medium Wells	OD Solvent Wells	% Survival in Solvent
24h 3/19/02	0.502	0.474	94.5%
	0.441	0.394	89.4%
48h 3/19/02	0.587	0.536	91.4%
	0.582	0.545	93.6%
72h 3/19/02	0.687	0.601	87.6%
	0.666	0.588	88.3%

The average survival in 0.5% DMSO from Table 2 was 90.8%.

II. Doubling Time Experiments

We ran a series of experiments designed primarily to determine the appropriate original seeding density for 24, 48, and 72 h exposure times. We judged our results on visual observations of the cells at the conclusion of the experiment (control cells should be just confluent at 24, 48, or 72h), and on the shape of the growth curve.

Figure 1.

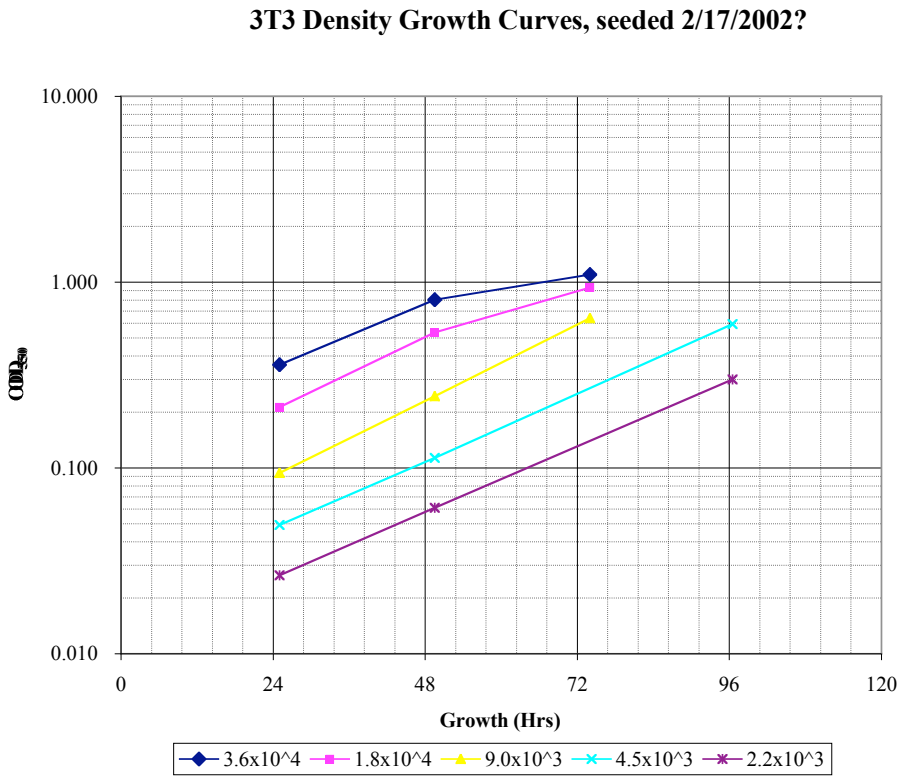
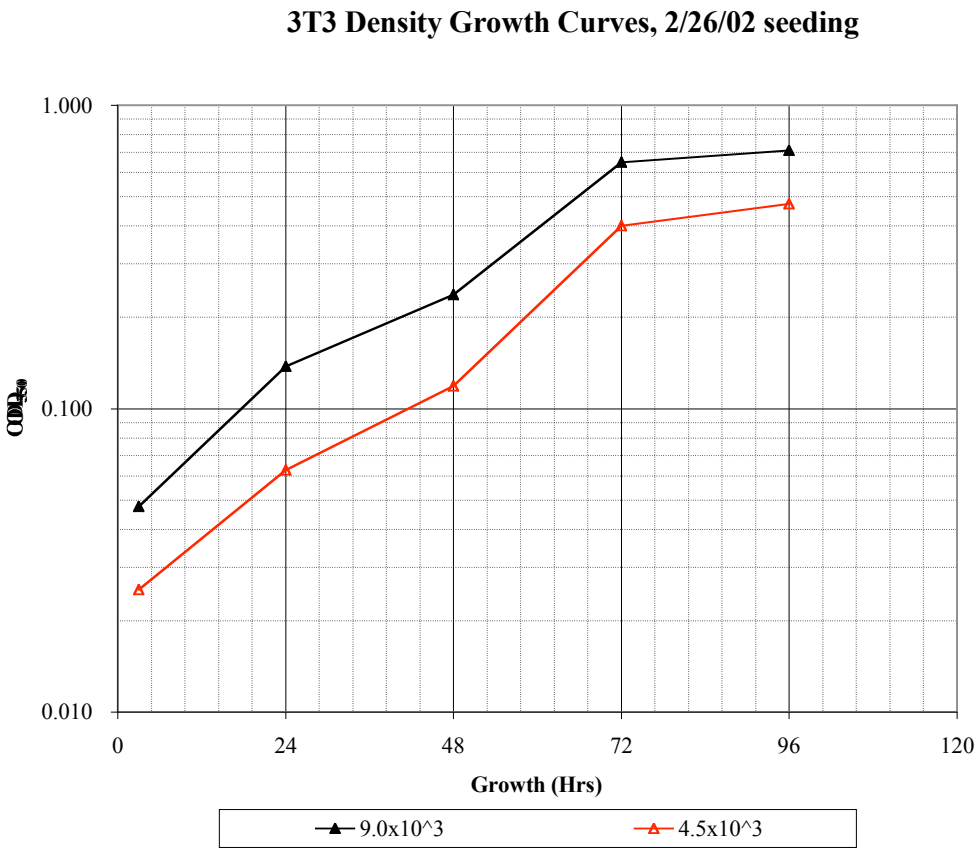


Figure 2.



We have concluded from these growth curves that our 3T3 cells have a doubling time of about 19 h and that cell concentration of: 1X104 cells/ml (24h); 5X103 cells/ml (48h); and 2.5X103 (72h) are acceptable.

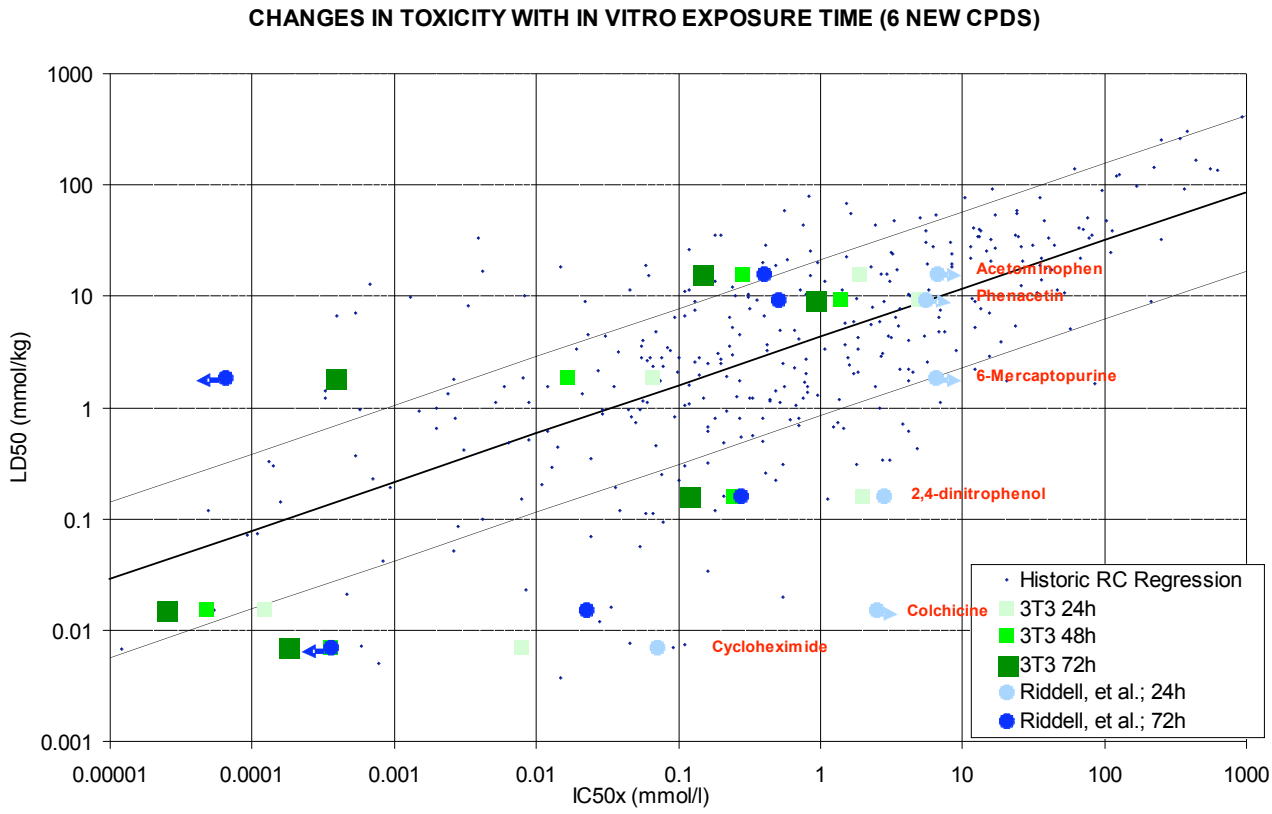
III. Exposure Duration

The exposure question was first raised by Richard Clothier who indicated that a paper by Riddell, et al. showed a number of chemicals whose toxicity changed greatly between a 24 h and a 72 h exposure (for 25/50 materials there was little change and for 25/50 materials there was a change). We examined the paper and chose to investigate six chemicals that showed some of the largest differences between 24h and 72h.

Our initial studies gave similar results to those of Riddell et al. However we felt that the cell number for the longer exposures was not optimal, and we conducted additional studies to determine a standard seeding density for each exposure period. Using this methodology we looked at the 6 materials in a standardized fashion at 24, 48 and 72h.

Our results are shown in Fig. 3.

Figure 3.



In this figure the historic Halle, et al. data are shown as small blue dots and the regression line as a dark black line. To add perspective we have included the Riddell, et al. data as a light blue diamond (24h) or a dark blue diamond (72h). Arrows emerging from certain points indicate that the value is less than or greater than that point. Our values are graphed in increasing shades of green from light (24h) to dark (72h). All green values are averages of at least two separate experiments. It appears that our data are somewhat different than Riddell, et al., i.e most differences are not as great as originally seen. Nonetheless the values, as expected, do become more toxic with increased exposure time. We feel that 48 hrs is probably the optimal time for these data if the Halle regression is considered some type of a standard.

Next we asked whether a 48 h exposure time would affect our earlier results with the 11 chemicals presented in the Guidance Document. If these numbers were changed significantly, this might cause us to make significant modification to our guidance.

To assess the effect of increasing exposure time on the 11 chemicals, we tested them with exposure times of 24h, 48h and 72h as shown in Fig. 4.

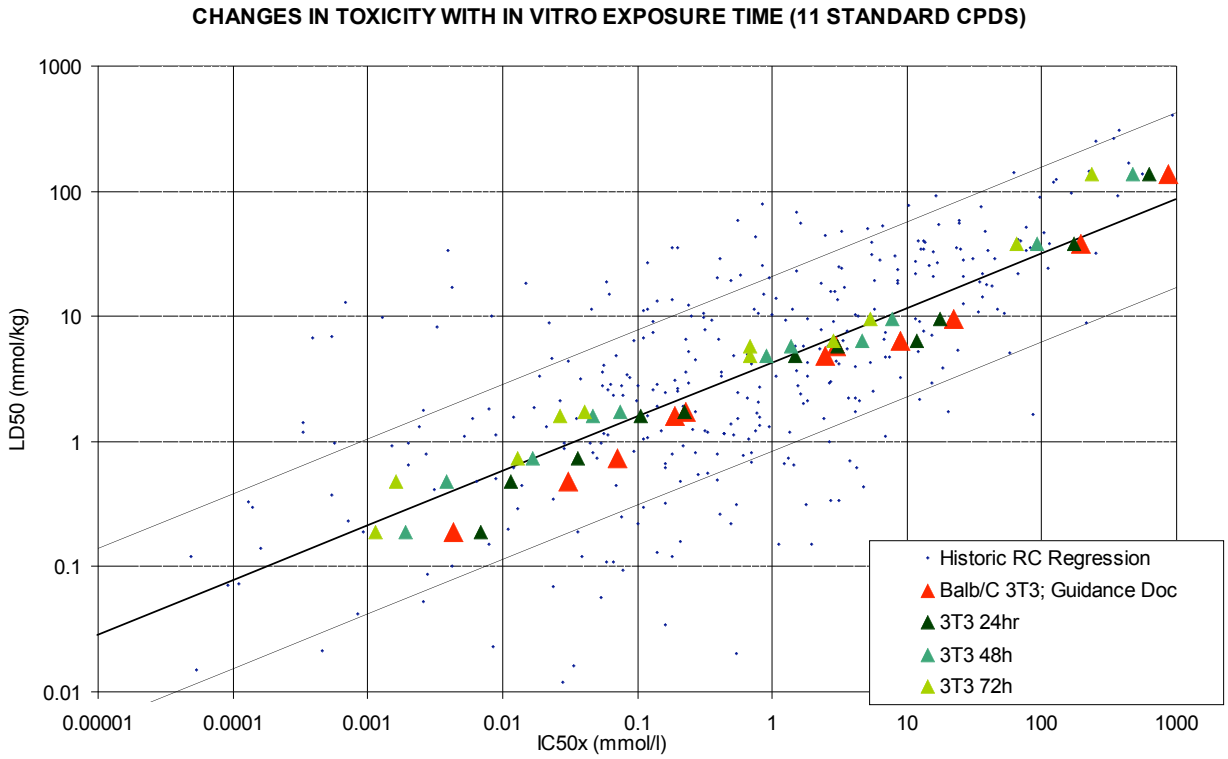
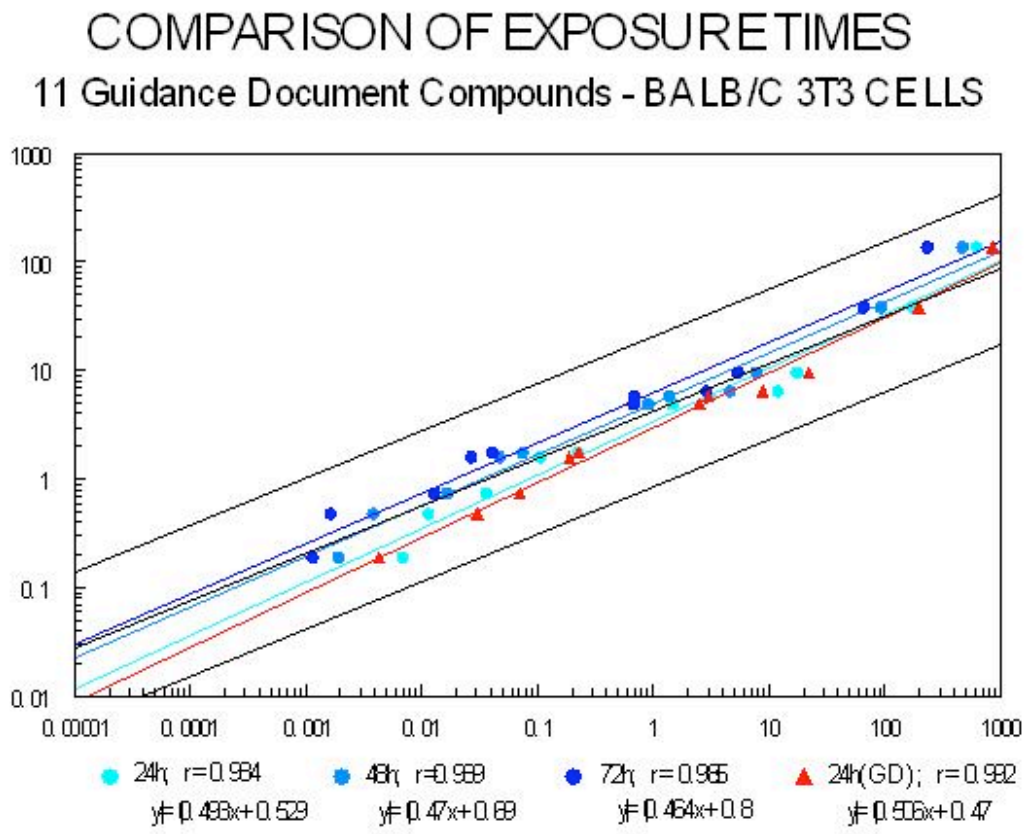


Figure 4.

The data shown on the graph are averages of duplicate experiments. It can be seen that although each of the chemicals becomes more toxic with increased exposure, all points are still within the 0.5 log range of the regression line. It again appears that 48 h exposure fits the regression more closely, however we regraphed the data in Fig. 5 to show the regression line and statistics for each of the new sets of data.

Figure 5.



In this figure it can be seen that all the regression lines for the 3 new time points plus the Guidance Document data (red triangles) fall with in the regression boundaries. It again appears that the 48 hour values best fit the original regression line.

We now feel that for the 3T3 cells an extended exposure period (>24h) should be used, and that 48h seems to help identify the more toxic compounds while not over estimating the less toxic ones.